2. Tunable symmetry in confined colloidal liquid crystals

Modern display technology relies on molecular liquid crystals, often confined to cells with boundaries that orientationally “anchor” the mesogenic molecules and control the optical properties of the cell. Biological molecules like virus rods and cytoskeletal polymers also exhibit liquid crystalline phases that are spatially confined. But in contrast to the liquid crystals used in displays, the sizes of these biopolymers are often on a similar length scale as the confinement. This raises the question how the finite length of the polymers influences the competition between bulk liquid crystalline ordering and boundary ordering. To answer this question, we confine nematic liquid crystals of monodisperse bacteriophage fd-virus rods inside shallow, donut-shaped microchambers. The doughnut geometry allows us to specifically address the question how colloidal nematics resolve topological constraints when singularities are no longer negligible in size. We quantify the nematic director fields by fluorescence time-lapse microscopy and custom-written image analysis software and compare the patterns with predictions of continuum theory as well as Monte Carlo simulations accounting for the finite particle size. We observe patterns that can be classified into categories based on their symmetries and defect patterns. Two of these patterns are expected on the basis of continuum theory, but one pattern—with a striking three-fold-symmetry—is predicted to occur only for rods of finite length. The remaining patterns may represent metastable states. At the end of the chapter, we also report first tests of packing of fd-rods in square chambers, where we find diagonal alignment of the rods, consistent with continuum theory. We propose that the interplay between bulk and surface ordering under
ultra-confined conditions can lead to novel liquid crystals with tunable symmetry.

2.1 Introduction

Modern display technology would be unthinkable without nematic liquid crystals (Castellano, 2005). This fluid phase of matter differs from typical liquids by exhibiting orientational molecular order, which affects the polarization of light (Sluckin et al., 2003). Liquid crystalline materials may have various types of molecular structure, but they have in common that the molecules interact via anisotropic attractive and repulsive forces. Often, the molecules are rod-shaped. Liquid crystalline phase transitions can be well-modeled by continuum theories such as the phenomenological theory of Landau and de Gennes (de Gennes and Prost, 1995), which account for the system’s change in symmetry. However, isotropic-nematic phase transitions have also been modeled by microscopic models (Singh and Dunmur, 2002), including the Maier-Saupe theory (Maier and Saupe, 1958) that attributes nematic ordering to anisotropic attractive interactions and the Onsager theory that considers exclusively repulsive interactions (Onsager, 1949).

Most applications of liquid crystals require these materials to be confined to cells with boundaries specifically treated to orientationally “anchor” the mesogenic molecules, allowing the optical properties of the cell to be controlled. The equilibrium director field is now determined by a competition between the mutual packing of the molecules and the surface anchoring conditions. This competition can lead to an interesting range of defect patterns when thermotropic nematics are confined in thin spherical shells (Fernandez-Nieves et al., 2007; Lopez-Leon et al., 2011; 2012). In shallow, square microchambers and in channels, molecular liquid crystals can adopt bi-stabile director fields, with two distinct, stable alignment states (Davidson et al., 2010; Tsakonas et al., 2007). Such bistability is advantageous for display applications, since it
allows one to use simply a pulse voltage to switch between “on” and “off” states.

Confinement-mediated ordering of molecular liquid crystals is commonly described by Frank elastic theory and Landau-de Gennes theory (de Gennes and Prost, 1995; Majumdar et al., 2007; Tsakonas et al., 2007). These are continuum theories, which account for the competition between the elastic free energy of the liquid crystal and the surface anchoring imposed by the confinement. For low molecular weight mesogens, continuum descriptions are indeed well-suited, since the lengths of these molecules are in the nm-range, well below the micron-sized dimensions of typical confining geometries (for reference, current state-of-the-art in displays employ ~70 μm pixels). Nature, however, provides us with many examples of large-molecular weight mesogens that measure several microns in length, such as actin and microtubules (Bras et al., 1998; Buxbaum et al., 1987; Coppin and Leavis, 1992; Furukawa et al., 1993; Suzuki et al., 1991), DNA (Strzelecka et al., 1988), and rod-like viruses (Bawden et al., 1936; Bernal and Fankuchen, 1941; Dogic and Fraden, 2006; Fraden et al., 1989; Lapointe and Marvin, 1973). Often, these rod-like polymers are confined in cellular (sub)volumes with micron-sized dimensions, comparable to the contour length and persistence length of the particles themselves. Actin and microtubules are for instance packed within cellular compartments such as lamellipodia and filopodia (Medalia et al., 2002) and DNA is packed at high density inside virus capsules (Speir and Johnson, 2012). These strongly confined conditions raise the question how the competition between mutual packing interactions and confinement play out when both effects share the same length scale.

Mutual packing interactions in bulk liquid crystals of colloidal rods are well-understood experimentally and theoretically. For rods interacting exclusively through hard-core repulsive interactions, the isotropic-nematic phase transition is usually described by models based on seminal theory by Onsager (Onsager, 1949). For hard rods, the free energy of the system consists only of entropic terms, in particular the orientational and translational entropy of the molecules. These two terms compete, since orientational entropy is maximized when the rods point in all directions with equal probability, whereas translational entropy is maximized when rods are aligned, since alignment maximizes
the total free volume in which rods can diffuse. At low rod volume fractions, rotational entropy dominates and the suspension is isotropic, but above a critical volume fraction, excluded-volume (or packing) entropy dominates and the orientations of the rods are distributed about a preferred direction. Onsager considered the limiting case where the ratio of rod length $L$ over diameter $D$ tends to infinity. In this case, the I-N phase transition may be treated with a virial expansion of the free energy truncated at the second virial coefficient. The entropic transition from an isotropic to a nematic state is predicted to take place at a critical volume fraction $\phi_{\text{crit}} = 3.340 \frac{D}{L}$. This prediction is exact in the limit of infinite aspect ratio, but remains surprisingly accurate for aspect ratios down to about 100 (Frenkel, 1987). For shorter rods, high-order virial coefficients become increasingly important (Bolhuis and Frenkel, 1997).

Modifications of the Onsager theory exist, for instance to account for rod semi-flexibility (Chen, 1993; Khokhlov, 1982), the soft repulsive interactions between charged rods (Stroobants et al., 1986), attractive van der Waals interactions (van der Schoot and Odijk, 1992), and polydispersity in rod length and diameter (Odijk, 1986; Vroege and Lekkerkerker, 1992).

The Onsager theory describes orientational ordering in bulk (unconfined) liquid crystals. It is much less well understood what happens when confining walls pose additional packing constraints on liquid crystals of finite-sized particles. Continuum theories have been applied, but these do not account for the finite size of the rods (Galanis et al., 2006; 2010b; Vitelli and Nelson, 2006). Numerical Monte-Carlo simulations have been reported to describe ordering of nematic fluids of colloidal rods in spherical cavities and shells (Dzubiella et al., 2000) and wetting of these suspensions at planar or curved hard walls (Dijkstra et al., 2001; Holyst and Poniewierski, 1987; Poniewierski and Holyst, 1988; van Roij et al., 2000). However, these simulations again did not directly address the question of how finite rod size influences the competition between bulk and surface ordering.

Meanwhile, experiments on confined colloidal rods have mostly addressed dilute suspensions. Individual biopolymers have been shown to orient along the longest axis of microchambers (Cosentino Lagomarsino et al., 2007) due to a competition between configurational entropy and bending enthalpy. For the same reason, isotropic entangled
networks of actin filaments organize into peripheral shells when confined in spherical droplets and vesicles (Claessens et al., 2006a; Limozin et al., 2003). Confinement of nematic phases has been reported for actin filaments in microchambers (Deshpande and Pfohl, 2012; Soares e Silva et al., 2011a) and fd-virus rods in wedges (Dammone et al., 2012). However, these studies again did not directly address the question how finite rod size influences the competition between bulk and surface ordering.

Here we investigate the director fields of colloidal nematic liquid crystals in shallow, circular microwells whose diameters are comparable to the rod length. We use rod-shaped bacteriophage fd-virus as a model liquid crystal system, since these rods are monodisperse, their interactions are hard-core like when the charges are screened, and their phase behavior in bulk is well-known (Dogic and Fraden, 2000; Dogic et al., 2004; Purdy and Fraden, 2004; Purdy et al., 2003; Tombolato et al., 2006). We use standard photolithography to produce non-adhesive doughnut-shaped SU-8 chambers whose overall diameter and width of the annulus is varied. This doughnut shape allows us to specifically address the question how colloidal nematics resolve topological constraints when singularities are no longer negligible in size.

In Section 2, we briefly summarize theoretical results of numerical simulations performed by Pieter Mulder, Ioana Garlea, and Bela Mulder (Mulder, 2012). These simulations predict liquid crystals with special symmetries that emerge as a consequence of the finite-size of the rods. In Section 3, we present director fields obtained experimentally by performing confocal time-lapse imaging of a low density of fluorescently labeled rods in the nematic background of unlabeled rods. Since fd-rods have a length of 0.88 µm, diameter of 6.6 nm ($L / D \sim 130$), and persistence length of 2.2 µm, we can observe individual virus particles and their anisotropic (mostly axial) diffusion in the nematic background (Lettinga et al., 2007). To compute director fields, we analyze the time-averaged orientations of the rods by custom-written software. We confirm the existence of the special symmetries predicted by numerical simulations, in particular the occurrence of a liquid crystal pattern with three-fold symmetry that is a direct result of the finite length of the rods. We propose that the interplay between optimal packing and confinement could lead to novel liquid crystals with tunable symmetry.
2.2 Theory

In order to investigate the optimal packing of colloidal rods in confined spaces, we examine the following problem: consider a suspension of stiff, thermally agitated rods of length $L$ in a nematic liquid crystal phase (Fig. 1a). The rod orientations are distributed around one preferred direction known as the nematic director, but the rod positions are disordered. Next, consider these rods in a two-dimensional, annular chamber of outer diameter $d_o$ and inner (or hole) diameter $d_i$. Wall-anchoring results in rods aligning parallel to the circular contours of the edges of the chamber. As long as the rod length $L$ is much smaller than both the chamber and the hole diameter, this boundary condition establishes a nematic field devoid of defects, exhibiting infinite-fold rotational symmetry which we will denote as $U(1)$ (see Fig. 1b). However, when the hole is removed ($d_i = 0$), a topological problem arises in the center of the chamber: although wall-anchoring should dictate that rods follow the circular contour, this would result in a thermodynamically unfavorable $+1$ singularity at the center (Fig. 1c). Instead, the system tends towards a configuration with two $+\frac{1}{2}$ singularities located at polar opposites of the chamber (Fig. 1d). The director field thus exhibits two-fold rotational symmetry denoted as $D(2)$.

These two problems can be mapped to each other by taking the limit $d_i \to 0$. But in order to realize this limit, $d_i$ must eventually compare to the length $L$ of the rods. In such a regime, the small-rod assumption breaks down and continuum theory does not apply. We hypothesize that when transitioning from the infinite-fold symmetry state to the two-fold symmetry state, intermediate states occur which are somehow determined by the length scale $L$. Such intermediate states might possess symmetries of intermediate order, considering that an abrupt break in symmetry order (from infinite-fold to two-fold) occurs only when $d_i = 0$ is realized under the small rod assumption.

This hypothesis was tested in numerical Monte-Carlo simulations performed by Ioana Garlea and Pieter Mulder in the group of Bela Mulder (AMOLF). In these simulations, rigid spherocylinders of length $L$ and thickness $D$ diffuse in quasi-two-dimensional chambers of outer diameter $d_o$, inner (hole) diameter $d_i$, and vertical height $d_z$ (Fig. 2).
Rods interact with other rods and with chamber boundaries via hard-core steric repulsions.

**Rods align to form higher-order symmetric states.** For disk-shaped chambers without a hole, the simulations produce a two-fold symmetric state identical to that predicted by continuum theory (Fig. 2a, compare to Fig. 1d). Strikingly, introducing a hole with diameter $d_i$ results in higher-order symmetries. The smallest holes result in a liquid crystal with three-fold symmetry (Fig. 2b). Increasing the hole diameter

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**Figure 1.** Prediction of continuum theory of the effect of boundary conditions on orientational ordering of a two-dimensional nematic liquid crystal. a. Schematic of rod-shaped molecules of length $L$ in a nematic liquid-crystalline phase. b. The nematic liquid crystal organizes to follow boundaries and exhibit infinite-fold symmetry when confined to a donut-shaped chamber of outer diameter $d_o$ and inner (hole) diameter $d_i$. c. Removing the hole reveals a thermodynamically unfavorable +1 defect. d. Instead, the stable configuration in the disk-shaped chamber contains two $+\frac{1}{2}$ defects, resulting in two-fold rotational symmetry.
results in four-fold symmetry (Fig. 2c). Five-, six-, seven-, and eight-fold symmetries can also occur, depending on the hole diameter $d_i$ and vertical chamber height $d_z$ (see Fig. 4). These higher-order symmetries are not predicted to be thermodynamically favorable according to continuum theory, which neglects rod length. Because the simulations explicitly account for finite rod length, these higher-order symmetries are a direct consequence of the optimal packing of finite-sized rods in a confined, annular geometry.

**Geometric correspondence occurs in 2D chambers.** Thin chambers whose vertical height $d_z$ equals the rod thickness $D$ exhibit particularly interesting behavior. These shallow chambers confine rods to a two-dimensional environment by completely suppressing out-of-plane rotations. In this case, the relationship between hole diameter $d_i$ and rod length $L$ controls the symmetry order. For size ratios $d_i / L = 3^{1/2} = 0.58$, rods can form an equilateral triangle around the hole; concomitantly, a three-fold symmetric pattern emerges for $d_i / L \approx 0.58$ (Fig. 3a). For
\[
\frac{d_i}{L} = 1, \quad \text{rods can form a square around the hole; concomitantly, a four-fold symmetric pattern emerges for } \frac{d_i}{L} \approx 1 \text{ (Fig. 3b). More generally, the number } n \text{ of rods that can form a regular polygon around the hole, given rod length } L \text{ and inner diameter } d_i, \text{ is given by the formula }
\]

\[
\frac{d_i}{L} = \cot \left( \frac{\pi}{n} \right).
\]

We use the term geometric correspondence to refer to situations where the number of rods } n \text{ is equal to the symmetry order of the resulting nematic texture. As shown in Fig. 4, simulations (yellow circles) show that geometric correspondence (blue lines) also holds for higher-order symmetries.

**Thicker chambers result in lower-order symmetry.** Chambers with a larger vertical height relative to the rod diameter (\(d_z / D = 3 \) and 6) were also investigated by numerical simulations. As shown in Fig. 4, already at \(d_z = 3 \) \(D \), the symmetry order (green circles) is greatly reduced compared to values expected from geometric correspondence (blue lines). Increasing chamber thickness to \(d_z = 6 \) \(D \) (red circles) further decreases symmetry order. These results show that thicker chambers which do not fully prohibit out-of-plane rod orientations result in symmetries whose order can be far lower than that expected from geometric correspondence.
Figure 4. Dependence of observed symmetry order on chamber inner diameter, $d_i / L$ (where rod length $L$ is $15 \times$ rod diameter $D$). Circles denote simulations performed with different chamber heights ($d_z / D$) and rod volume fraction ($\varphi$), as indicated in the legend. Blue lines denote expected symmetry order based on geometric correspondence. Figure courtesy of P. Mulder, I. Garlea, and B. Mulder.

2.3 Results

Two-, three-, and infinite-fold symmetries occur depending on chamber shape. Based on the simulations, we anticipate that liquid crystals of finite-sized rods in annular geometries should exhibit symmetries that cannot be accounted for by continuum theory. In order to test this hypothesis, we prepare non-adhesive microchambers with a donut shape and fill them with suspensions of rod-like fd-virus in the nematic phase (Fig. 5). We systematically varied the chamber outer diameter $d_o$ (from 10 µm to 100 µm) and the diameter of the inner hole $d_i$ (from 0 to 0.7 times the outer diameter $d_o$). The chambers have vertical heights in the range of $d_z = 1–3$ µm, which is the minimal thickness that was experimentally attainable. We note that this chamber thickness is still much larger than the diameter of the fd-rods, 6.6 nm (Dogic and Fraden, 2006). Nevertheless, time-lapse imaging of fluorescently...
labeled particles in the otherwise unlabeled background nematic phase showed that particles diffused mostly in-plane. Additionally, 3D z-scans confirmed that the cholesteric twist normally observed in bulk nematic phases of fd-rods (Dogic and Fraden, 2000; Grelet and Fraden, 2003; Purdy and Fraden, 2004; Tang and Fraden, 1995) was suppressed. To examine the nematic patterning, we acquired time-lapse image series of chambers containing fd-rods with a small tracer fraction that was fluorescently labeled. We developed a customized image analysis program (see Image Analysis) to quantify the orientation of the nematic director for each image pixel. By visual inspection of the rotational symmetry of the resulting nematic director fields and the location and number of defects, we categorize the patterns into 7 different types.

One commonly observed pattern is a nematic pattern with infinite-fold rotational symmetry that does not exhibit any obvious singularities (Fig. 6a). A second pattern is one with three-fold rotational symmetry, showing three \( +\frac{1}{2} \) singularities (Fig. 6b). A third pattern is one with two-fold rotational symmetry, showing two \( +\frac{1}{2} \) singularities at

![Figure 5](image_url)

**Figure 5.** The experimental model system. **a.** Image of a glass substrate patterned with SU-8 microchambers, acquired by scanning-electron microscopy. Scale bar 200 µm. **b.** Close up of the region indicated by the black box in panel a. Scale bar 20 µm. **c.** Fluorescence confocal image of an annular microchamber \( (d_o = 20 \, \mu m, \, d_i = 0.4 \, \mu m) \) filled with a suspension of partially labeled fd-virus rods (inverted grayscale lookup-table). Scale bar 5 µm. **d.** Image of a single fd-virus rod, acquired by transmission electron microscopy (reprinted from (Dogic and Fraden, 2006)). The rod length is 0.88 µm and the diameter is 6.6 nm \( (L / D \sim 130) \).
Figure 6. Three types of experimentally observed nematic patterns. **Top row:** Representative instances of the three patterns. Brightness corresponds to a maximum intensity projection of 2000 frames. Color corresponds to the average orientation $<\theta>$ of the nematic director (calibration wheel, right). **Bottom row:** Schematic diagrams of the nematic pattern types, showing singularities as black points, further indicated by the arrows. **a.** A pattern of infinite-fold symmetry in narrow annular chambers exhibiting no singularities. **b.** A pattern of three-fold symmetry in wider annular chambers exhibiting three singularities. **c.** A pattern of two-fold symmetry in disc-shaped chambers exhibiting two singularities.

Two opposite poles (Fig. 6c). The first and third patterns agree well with continuum theory (cf. Figs 6a,c and 1b,d). The second pattern is not predicted by continuum theory, but is consistent with the three-fold-symmetrical state predicted by the simulations (cf. Figs 6b and 2b). The simulated pattern arose from the finite size of diffusing rods, suggesting that the three-fold symmetric pattern we observe in experiment is a direct consequence of the finite length of the fd-rods. The occurrence of the observed nematic patterns depends strongly on the shape and dimensions of the confining chambers, as shown in Fig. 7, where the probability of occurrence of the three nematic patterns (see Methods) is plotted against the inner hole diameter $d_i$ (in units of outer diameter $d_o$).

The pattern with infinite-fold symmetry is most likely to occur in narrow ring-shaped chambers with a large hole in the middle with
We denote this pattern with the symbol $U(1)$, which corresponds to the infinite-fold rotational symmetry of the unit circle given by the unitary group of degree 1 (Cornwell, 1984) (Fig. 8a). Two typical examples of the $U(1)$ pattern are shown in Fig. 8b. The pattern occurs exclusively in the smallest chambers with diameters up to 50 µm (Fig. 8d).

The two-fold symmetric pattern with two $±\frac{1}{2}$ singularities on opposite ends of the chamber is most probable in disk-shaped chambers without a central hole ($d_i / d_o = 0$, see green diamonds in Fig. 7, see also Fig. 9b). We denote this pattern with the symbol $D_2$, which corresponds to the two-fold rotational symmetry of the dihedral group of order two (Atkins and de Paula, 2009) (Fig. 9a). $D_2$ patterns do form also in chambers with a hole ($d_i / d_o > 0$), but with a probability that decreases sharply with increasing hole size (Fig. 9c). The occurrence of the $D_2$ pattern exclusively in the smallest chambers with diameters up to 50 µm (Fig. 8d).
Figure 8

Figure 9
pattern also depends on the chamber size, decreasing with increasing $d_o$ and being zero for the largest chambers of 100 μm (Fig. 9d).

The three-fold symmetric pattern only occurs for chambers with a small but finite hole size ($d_i / d_o = 0.2$, red triangles in Fig. 7, see also Fig. 10c). This pattern has three evenly spaced $+\frac{1}{2}$ singularities (Fig. 10a). Two examples are shown in Fig. 10b. We denote this pattern $D_3$, which corresponds to the three-fold rotational symmetry of the dihedral group of order three. Hole sizes $d_i$ for chambers with $D_3$ patterns varied in the range 2–6 μm, corresponding to 1 to 3 fd-rod lengths. Furthermore, $D_3$ occurred mainly for smaller chambers up to 30 μm (Fig. 10d).

**Alternative nematic patterns.** So far we focused on only three out of the seven different nematic patterns we observed for confined fd-rod liquid crystals. The additional four nematic patterns, which we will describe below, are not predicted by either the continuum theory (which predicts 2 patterns) or the simulations (which predict only three patterns in the parameter range that we used). Here we describe each pattern type and quantify their probability of occurrence as a function of both outer diameter and inner (hole) diameter.

**N occurs for small chambers.** In the smallest chambers (outer diameter $d_o = 10$ μm) we observed nematic liquid crystals where rods aligned with each other but not along the circular contour of the chamber (Fig. 11a,b). We denote this pattern, which resembles a bulk

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**Figure 8.** Pattern $U(1)$. a. Schematic. b. Representative instances of $U(1)$. Brightness corresponds to maximum intensity projection of 2000 frames. Color corresponds to the average orientation $<\theta>$ of the nematic director (calibration wheel, right). Scale bars 5 μm. c-d. Probability of occurrence of $U(1)$, given inner diameter $d_i$ (c) and outer diameter $d_o$ (d).

**Figure 9.** Pattern $D_2$. a. Schematic. b. Representative instances of $D_2$. Brightness corresponds to maximum intensity projection of 2000 frames. Color corresponds to the average orientation $<\theta>$ of the nematic director (calibration wheel, right). Scale bars 5 μm. c-d. Probability of occurrence of $D_2$, given inner diameter $d_i$ (c) and outer diameter $d_o$ (d).
Figure 10

Figure 11
nematic state, with the symbol \( N \). These patterns occur over a broad range of hole sizes (Fig. 11c), but occur only when \( \bar{d}_o = 10 \mu m \) (Fig. 11d). \( N \) patterns may be related to the \( D_2 \) patterns, in that we anticipate that there are two opposite singularities displaced at a distance \( x \) beyond the chamber edge (Fig. 11e).

**Asymmetric \( S \) patterns.** Asymmetric patterns were also observed. Some chambers exhibited only one singularity (Fig. 12a,b). Because of the lack of non-trivial rotational symmetry, we denote this pattern \( S_1 \) for “one singularity”. This pattern was mostly observed in thin, ring-like chambers (\( \bar{d}_i / \bar{d}_o = 0.5 \) and 0.7, Fig. 12c). The probability of finding \( S_1 \) increases somewhat with increasing chamber size, but is observed over the entire range of chamber sizes (10-100 \( \mu m \)) (Fig. 12d).

Another asymmetric pattern exhibited two singularities, similar to \( D_2 \), but unlike in the \( D_2 \) pattern, the defects were not positioned at polar opposites (Fig. 13a,b) We call this pattern \( S_2 \), which denotes a pattern with two singularities exhibiting no particular rotational symmetry. This pattern occurred over a wide range of hole and chamber sizes with no particular preference (Fig. 13c,d). We quantify the relative angular positions of the two singularities for \( S_2 \) patterns by the angle \( \beta \) (Fig. 13e), and find a broad angle distribution ranging from 90 to 160°, with a peak

**Figure 10.** Pattern \( D_3 \). a. Schematic. b. Representative instances of \( D_3 \). Brightness corresponds to maximum intensity projection of 2000 frames. Color corresponds to the average orientation \( \langle \theta \rangle \) of the nematic director (calibration wheel, right). Scale bars 5 \( \mu m \). c-d. Probability of occurrence of \( D_3 \), given inner diameter \( \bar{d}_i \) (c) and outer diameter \( \bar{d}_o \) (d).

**Figure 11.** Pattern \( N \). a. Schematic. b. Representative instances of \( N \). Brightness corresponds to maximum intensity projection of 30 frames. Color corresponds to the average orientation \( \langle \theta \rangle \) of the nematic director (calibration wheel, right). Scale bars 5 \( \mu m \). c-d. Probability of occurrence of \( N \), given inner diameter \( \bar{d}_i \) (c) and outer diameter \( \bar{d}_o \) (d). e. Schematic of hypothetical point defects displaced beyond the chamber edge by a distance \( x \).
Figure 12. Pattern $S_1$. a. Schematic. b. Representative instances of $S_1$. Brightness corresponds to maximum intensity projection of 2000 frames (left) and 30 frames (right). Color corresponds to the average orientation $\langle \theta \rangle$ of the nematic director (calibration wheel, right). Scale bars 5 µm. c-d. Probability of occurrence of $S_1$, given inner diameter $d_i$ (c) and outer diameter $d_o$ (d).

at $\beta = 140^\circ$ (Fig. 13f). (Note that this distribution excludes chambers which have been classified $D_2$ and therefore exhibit $\beta = 180^\circ$.)

**Disordered B patterns occur in large chambers.** Sometimes, multiple singularities were scattered across the interior of the chamber, and the nematic director field exhibited chaotic-looking patterns (Fig. 14a,b). We denote such structures with the symbol $B$. This type of pattern was mostly observed in chambers with small holes ($d_i/d_o = 0.1$, Fig. 14c) and in large chambers with $d_o > 50$ µm (Fig. 14d).
Figure 13. Pattern $S_2$. a. Schematic. b. Representative instances of $S_2$. Brightness corresponds to maximum intensity projection of 2000 frames. Color corresponds to the average orientation $\langle \theta \rangle$ of the nematic director (calibration wheel, right). Scale bars 5 µm. c-d. Probability of occurrence of $S_2$, given inner diameter $d_i$ (c) and outer diameter $d_o$ (d). e. Schematic representing the angle $\beta$ formed by the two point defects and the center of the circle. f. Histogram of observed values of $\beta$ for $S_2$ patterns.
2.4 Discussion

We confined nematic suspensions of fd-virus rods in shallow donut-shaped chambers and reported experimental evidence of a three-fold rotationally symmetric pattern which we denote $D_3$. We propose that this pattern arises from the optimal packing of confined, colloidal rods, whose sizes cannot be neglected. The main argument to support this interpretation is that the $D_3$ pattern is identified as a thermodynamically favorable configuration in simulations accounting for finite particle size, whereas it is not predicted by continuum theory. Moreover, the pattern exhibits intermediate properties that suggest it occurs in a transition from $U(1)$ to $D_2$. First, the three-fold symmetry of $D_3$ is of an order that lies between the two-fold symmetry of $D_2$ and the infinite-fold symmetry...
of $U(1)$. Second, $D_3$ was most likely in chambers with intermediate hole diameters $d_i / d_o = 0.2$, in between the hole diameters where $D_2 (d_i / d_o = 0)$ and $U(1) (d_i / d_o = 0.7)$ patterns were observed (cf. Fig. 7).

Although the existence of $D_3$ in experiment and simulation indicates consistency, experiment and simulation could not be performed with identical parameters, as detailed in Figure 15. In particular, the confinement in the $z$-direction was much less stringent in the experiments (1.1–3.4 rod lengths) than in the simulations (1–6 rod diameters). This likely explains why $D_3$ patterns occurred for different values of $d_i / L$: in experiment, $D_3$ occurred for $d_i / L = 2.5–7.5$. These values are greater than expected from geometric correspondence (cf. Fig. 3), where we should expect $d_i / L \approx 3^{-1/2} = 0.58$. Experimentally, we cannot rule out that $D_3$ is not a thermodynamically favorable condition based solely on consistency with simulation data. However, simulation found that thicker (more three-dimensional) chambers exhibited lower-order symmetries (cf. Fig. 4). In particular, $D_3$ occurred over a broad range of parameters, up to $d_i / L \approx 1.7$, which approaches the experimentally observed interval of 2.5–7.5. The chambers investigated in simulation had vertical height $d_z$ that were at most equal to about half a rod length $L$, while in experiment the chamber height was 1.1–3.4 times the rod length $L$. It will be interesting to extend the computational work with calculations over a broader region of parameter space, to identify when $D_3$ is a thermodynamically favorable state. Furthermore, experiments on thicker colloidal rods such as microtubules may lead to the direct observation of higher-order symmetry states.

Despite differences in parameters, simulation and experiment furthermore agreed on the occurrence of $N$ patterns. In experiment, we found patterns that resembled $D_2$, but with expelled singularities (cf. Fig. 11). This $N$ pattern also occurred in simulation when rod length $L$ or density $\phi$ were increased (Mulder, 2012) (results not shown here). Prior theoretical work on colloidal rods in tactoidal droplets, where two virtual defects occur outside the droplet, similarly observed a transition to a bipolar state where defects approached the droplet surface to become true defects as droplet size increased (Prinsen and van der Schoot, 2004). These predictions agree well with experiment, where we find tactoid-like $N$ patterns for small outer diameters $d_o$. This agreement suggests
that the control parameter $d_0 / L$ determines a crossover between $D_2$ and $N$.

Further agreement between experiment and simulation occurred with the asymmetric pattern $S_2$. This pattern was also observed in simulation, albeit as a transient state while simulated systems were approaching equilibrium (Garlea and Mulder, 2013). Simulated systems that exhibited transient $S_2$ patterns ultimately equilibrated to $D_3$ symmetries. The two singularities in $S_2$ occurred at two vertices of an equilateral triangle. This observation roughly agrees with experiment: we found a wide distribution of angles $\beta$ with a peak at $\beta = 140^\circ$. This experimental value is in reasonable agreement with the value of $\beta = 120^\circ$, which corresponds to the intermediate state observed in simulations. This suggests that experimental $S$ patterns may represent a metastable precursor to $D$-type symmetries.

Figure 15. Comparison of length scales probed in simulations and experiments. a. Schematic of an annular chamber with outer diameter $d_o$, inner diameter $d_i$, and vertical height $d_z$. b. Schematic of rods with thickness $D$ and length $L$. c. Table comparing values of $d_o, d_i, d_z, D$, and $L$ between simulation and experiment.
This hypothesis raises the important question whether the experimentally observed patterns represent thermodynamic equilibrium. Although we cannot strictly rule out the possibility of nonequilibrium behavior, the nematic patterns we observed were stable over the course of 1–24 h after sample assembly and were reproducible over a large number of chambers. Furthermore, many of the experimental patterns correspond to predicted equilibrium configurations by simulations. Interestingly, $U(1)$, $D$, $N$, and $S_2$ patterns were most likely in small chambers ($d_o \leq 30 \mu m$, cf. panel d of Figs 8, 9, 10, 11, and 13), whereas the potentially metastable $B$ and $S_1$ patterns were most likely in large chambers ($d_o \geq 50 \mu m$, cf. panel d of Figs 12 and 14). This observation suggests that smaller chambers may be more likely to produce equilibrium structures.

### 2.5 Outlook: Square Chambers

So far we investigated the breakdown of continuum theory arising from the optimal packing of hard rods of finite size in annular geometries. Previous experiments with actin filaments (Soares e Silva et al., 2011a) and vibrofluidized rods (Galanis et al., 2006; 2010a) suggest that finite-size effects also influence packing of rods in microchambers with a different shape, such as square chambers. Here we report first tests of packing of fd-rods in square chambers and examine how finite-rod-size effects could manifest in these geometries. Continuum theory predicts that rods close to the chamber edges align along the walls, while rods at the center align along the long axis of the chamber, which is one of the two diagonals (Fig. 15a). The competition between bulk and surface ordering leads to diagonal alignment, with splay deformations in two diagonally opposing corners, and bend deformations in the other two opposing corners.

Recent simulations by Ioana Garlea and Bela Mulder address the limit of finite rod size. Similar to the simulations summarized in Section
2, hard rods diffuse in boxes with dimensions $d_z = 3D$ and $d_x = d_y = 73D$, where $D$ is the rod diameter. As before, box vertical height $d_z$ was chosen such that rods cannot freely rotate out of the $xy$-plane. In sharp contrast to continuum theory, the simulations predict that the liquid crystal adopts a line in place of a bend deformation, since the rods are unable to bend. At the line defect, the orientations of the rods change in a discontinuous manner (Fig. 16b).

To test whether such line defects can be observed experimentally, we confined nematic liquid crystals of fd-virus rods in shallow square- and rectangular-shaped chambers. As in Section 3, we acquire time-lapse image series of chambers containing fd-rods with a small tracer fraction that was fluorescently labeled. Again, we quantify the orientation of the nematic director using the customized image analysis routine described in Section 7. The nematic field director closely resembles predictions from continuum theory, with parallel alignment of rods near the walls and diagonal alignment in the chamber center (Fig. 16c). This alignment also resembles the patterning we find for nematic liquid crystals of actin filaments (Chapter 2). However, the data do not clearly reveal a sharp discontinuity in rod orientation at the corners, as predicted by the finite rod simulations. Because the line defect in simulation was predicted to have a size on the order of the rod length $L$, the discontinuity should manifest itself on length scales of $0.88 \mu m$ for fd-virus rods. In principle, a discontinuity of this length scale should be detectable. However, the square chambers in simulation have perfectly sharp corners, whereas the edges of the experimental chambers are rounded due to the limited ($\sim 1 \mu m$) resolution of the photolithographic techniques used to produce them. The rounded edges may assist in the formation of a continuous bend structure rather than a discontinuous line defect. Future experiments which employ either chambers with sharper corners (for instance made by electron beam lithography), or suspensions of longer rods (for instance actin or microtubules), should be capable of experimentally verifying the line defect predicted by simulations of diffusing rods in square chambers.
2.6 Methods

**Bacteriophage fd-virus preparation.** fd-virus rods were grown using a standard protocol (Dogic and Fraden, 2005) and stored in fd-buffer (20 mM tris pH 8.15, 100 mM sodium chloride, 15% ethanol). Assay suspensions were prepared at concentrations of 24 mg mL$^{-1}$, slightly above the bulk isotropic-nematic biphasic region, which occurs at ~20 mg mL$^{-1}$, in agreement with Onsager theory (Tang and Fraden, 1995). Suspensions were biphasic, as evidenced by visual inspection through crossed polarizers. fd-rods have a molecular weight of $1.64 \cdot 10^7$ Da (Zimmermann et al., 1986). We chose the lowest possible nematic concentration, in order to minimize the energy cost to re-arrange from one director field to another and thus reduce the probability of getting stuck in high energy metastable states. Fluorescently labeled rods were prepared by incubation with Alexa-488 succinimidyl ester (Dammone et al., 2012) (Invitrogen). A small amount of labeled rods (2–4% v/v)
was mixed with unlabeled rods in order to make individual labeled rods distinguishable by fluorescence microscopy.

**Microchamber preparation.** Microchambers were assembled using a standard photolithographic technique. In short, glass cover slips were spin-coated with a layer of photoresist and exposed to UV light patterned by a customized mask. Before spin-coating, cover slips (thickness #1, Menzel Gläser) were cleaned with base piranha (water, 30% ammonium hydroxide, 30% hydrogen peroxide at a ratio of 5:1:1; 75 °C for 15 min), followed by rinsing with water and baking (200 °C, 5 min). Cover slips were spin-coated (Delta 80 GYRSET, SUSS MicroTec; spin speed 3000 rpm) with a negative photoresist (SU-8 2005, MicroChem). Layer thickness was adjusted by diluting photoresist with cyclopentanone by approximately 10–20% and measured by a profilometer (Alpha-Step 500, KLA-Tencor). Coated cover slips were then baked (95 °C, 5 min) before exposure to ultraviolet light (only wavelengths above 365 nm, BG-12 bandpass filter, Schott) in a mask aligner (MJB, Karl Süss; typical dosage: 50–100 mJ cm⁻²). Patterning was achieved through custom-designed mask of chromium features printed on soda-lime glass (DeltaMask). The mask design included circular and annular geometries with outer diameters \(d_o\) of 10, 20, 30, 50, 70, and 100 µm. For each outer diameter, geometries with inner diameters \(d_i\) of 0, 0.1, 0.2, 0.3, 0.5, and 0.7 times the outer diameter \(d_o\) were made, resulting in 36 different geometries.

Exposed cover slips were baked (95 °C, 5 min), developed (2-methoxy-1-methylethyl acetate, MicroChem; 1–2 min), rinsed with isopropanol, and ultimately hard-baked (150°C, 2h). This process results in a glass substrate with a layer of photoresist patterns that form the basis of microchambers. Next, microscope slides (Menzel Gläser) were coated with a layer (~1 mm thick) of polydimethylsiloxane rubber (Sylgard 184, Dow Corning; 10:1 base:curing-agent w/w ratio; 120 °C, 5 min). Rubber-coated glass was rendered hydrophilic by corona discharge (BD-20V high-frequency generator, Electro-Technic Products) and soaked overnight in fd-buffer containing 0.1 wt% of the amphiphilic block copolymer Pluronic F-127 (Sigma-Aldrich), which effectively blocked nonspecific adsorption of fd-rods as confirmed by time-lapse imaging of rod diffusion. Saturation of the PDMS with buffer prevented drying of the sample for at least 24 hours.
We used the same lithography technique to prepare rectangular microwells with short edges of length 2–50 µm and aspect ratios of 1 to 18. We used chamber heights of 0.7, 3 and 5 µm. However, the cells of height 0.7 µm were difficult to fill, thus we used 3 µm for the majority of the experiments.

**Confinement assay.** A drop of fd-virus suspensions was placed on a glass-photoresist substrate and pressed against rubber-coated glass to form microchambers. After hermetically sealing glass edges with VALAP (equal parts vaseline, lanolin, and paraffin wax), samples were left to equilibrate for at least 30 mins and subsequently visualized by fluorescence microscopy. Only chambers that were well-sealed were considered, amounting to approximately 40% of all chambers. Chambers that were not well-sealed were evident by diffusion of fluorescently labeled rods escaping from the chamber (Fig. 17). The rods rapidly organized in steady-state nematic patterns within 30 minutes after filling the chambers. When we filled the chambers with the isotropic phase below the biphasic region, a nematic was not formed and the sample remained isotropic. This observation implies that the filling (and confinement) were not sufficient to induce a nematic.

**Fluorescence microscopy.** Microchambers were visualized using two microscope setups: (1) a spinning disk confocal scanner (CSU 22, Yokogawa) on an inverted microscope (DMIRB, Leica) with a cooled, electron-multiplying charged-coupled device (C9100, Hamamatsu) and (2) a Nikon C1 confocal point scanner on an inverted microscope (Ti, Nikon) with a photomultiplier tube detector (A1, Nikon). Labeled rods were excited with 488 nm laser light (Coherent). A series of images were recorded over a long enough time interval such that viruses diffused across the entire chamber. The average diffusion constant of virus rods in the nematic phase is $D_{||} \approx 1 \mu m^2/ s$ along the nematic director and $D_{\perp} \approx 0.1 \mu m^2/ s$ perpendicular to the nematic director (Lettinga et al., 2007). These diffusion constants result in diffusion timescales of tens of seconds for diffusion by one particle length and minutes for diffusing over inter-particle distances between fluorescently labeled rods (~ a few µm). The anisotropy in diffusion constants is apparent in Figure 18, where tracks of fluorescently labeled rods are clearly elongated along the direction of the nematic director. For spinning disk data, ~ten movies of 200 frames each were acquired at a fast imaging rate (0.1 frames per
second), which were separated by 2 minutes to allow rods to diffuse completely across the chamber (Fig. 18). For point-scanning confocal data, ~15–30 frames were acquired at a slower rate (1 frame per ~1–2 minutes) over several fields of view which were automatically acquired and stitched (NIS Elements, Nikon). A customized image analysis technique was developed to determine the average nematic director orientation $<\theta>$ given average orientations $\theta$ of labeled fd-rods across all images acquired (see Image Analysis).

Identification of nematic patterns. Rods formed a variety of liquid crystal patterns. Pattern type was determined by visual inspection of the nematic director fields of all well-filled chambers on the chip. Figure 19 summarizes the pattern frequency as a function of $d_o$ and $d_i/d_o$. We define the probability that a pattern $P$ occurs in a given chamber geometry $G$ as the number of observed instances of $P$ given $G$ divided by the total number of well-sealed chambers with geometry $G$ (Fig. 20). A total of 243 chambers were analyzed: 80, 27, 26, 36, 43, and 31 chambers for $d_i/d_o = 0, 0.1, 0.2, 0.3, 0.5,$ and 0.7 respectively; 77, 75, 55, 25, 9, and 2 chambers for $d_o/\mu m = 10, 20, 50, 70,$ and 100, respectively; 23, 113, 5, 21, 33, 32, and 16 chambers for patterns $U(1)$, $C_2$, $C_3$, $N$, $S_1$, $S_2$, and $B$, respectively.

Figure 17. Improperly sealed chambers are clearly evident by rods escaping chambers at the interface between the chip and the lid. Image represents a maximum time projection over 50 s. Escape events are visible as tracks of fluorescence that extend past chamber boundaries (white arrows). Scale bar 5 µm.
2.7 Image Analysis

We developed an algorithm where we quantify the orientation of the nematic director for each image pixel, given an image series (with \( N \) frames) of fluorescently labeled rods. In short, we determine rod orientation \( \theta \) for each frame, and average over frames to get the mean orientation \( \langle \theta \rangle \) per pixel.

**Step 1**: Given an image of fluorescent rods (Fig. 21a), we compute their orientation \( \theta \) (Fig. 21b) and the gradient energy, which quantifies the contrast between bright and dark pixels (Fig. 21c). This step is implemented using OrientationJ, which is a freely-available ImageJ plugin originally developed to track collagen and elastin fibers (Fonck et al., 2009; Rezakhanliha et al., 2012). This routine computes structure tensors constructed of the spatial gradients of fluorescence intensity around each pixel \((x,y)\). Determining the eigenvectors of a structure tensor yields the characteristic orientation \( \theta \) of the fluorescence intensity of a small region \((x\pm\sigma, y\pm\sigma)\) around each pixel \((x,y)\). We set the parameter \( \sigma = 3 \) px, which corresponds to the typical length scale of a
Figure 19. Frequency of occurrence over $d_o$-$d_i$ phase space. Black circles denote number of occurrences of a given pattern. Gray circles denote total number of chambers analyzed. Circle area depicts frequency (legend, top-right).
Figure 20. Probability of occurrence over $d_o$-$d_i$ phase space. Color denotes probability (legend, top-right), defined by the number of occurrences observed divided by the total number of chambers analyzed.
Furthermore, the trace of the structure tensor yields the gradient energy. Note that this quantity should not be confused with a physical energy. Rather, it is related to the notion of signal energy (Mitra and Kaiser, 1993).

**Step 2:** The energy image from Step 1 is thresholded (Fig. 21d) using Otsu’s method (Otsu, 1975). This yields a binary image comprising connected components of bright pixels (1) against a dark background (0). Bright pixels in this image correspond to points at or near a fluorescently labeled virus particle.

**Step 3:** The orientation image from Step 1 is masked using the threshold from Step 2 (Fig. 21e). The result is a set of orientation measurements $\theta$ only for pixels at or near a fluorescently labeled virus particle. We thus discard orientation measurements of background pixels.

**Step 4:** Steps 1–3 are repeated for the $N$ frames of the dataset (Fig. 21f,g). The result is a series of $N$ images produced by Step 3 (Fig. 21h,i).

**Step 5:** The average orientation $\langle \theta \rangle$ per pixel is determined given the $N$ images from Step 4 (Fig. 21j,k). Each pixel can have up to $N$ orientation measurements, depending on how often it passes the

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**Figure 21.** Algorithm for determining average orientation $\langle \theta \rangle$ of the nematic director given many measurements of rod orientation $\theta$ in each pixel. a. First frame of original data. b. Orientation output of OrientationJ. Color corresponds to orientation $\theta$ around each pixel (calibration wheel, below). c. Energy output of OrientationJ. Note that energy is highest where changes in fluorescence intensity are largest. d. Otsu threshold of the energy image (c). e. Masking the orientation image (b) with the threshold image (d). Note that color corresponds to orientation of virus rods in original image (a). f-i. Looping over all frames of a set of images. f,g: original data for frames 200 and 2000. h,i: Masked orientation images for frames 200 and 2000. j: Maximum time projection of the original data for all frames. k: Average orientation of all rods, $\langle \theta \rangle$, which gives the local direction of the nematic director in each image pixel.
Figure 21
Figure 22. The von Mises distribution, defined over a closed, periodic support. All curves shown represent distributions with the same mean angle \( \langle \alpha \rangle = 0 \) but varying values of the concentration \( \kappa \) (calibration bar, right).

Figure 23. Determining mean orientation \( \langle \theta \rangle \) from distributions of individual measurements \( \theta \). a. Mean orientation \( \langle \theta \rangle \), as in Figure 21, panel k. b. Concentration \( \kappa \) for each pixel corresponding to panel a, where color corresponds to values of \( \kappa \) as indicated in the calibration bar on the right. c. Number of orientation measurements \( n \) per pixel, as determined by the number of frames that a given pixel passes the threshold in Step 2. d. Histogram of orientation measurements for three pixels indicated by triangles, diamonds, and pentagons in panels a–c.
threshold from Step 2. Usually, background pixels never pass the threshold from Step 2 and therefore do not have orientation measurements.

The von Mises distribution. Computing the arithmetic mean is not a suitable method to determine average orientation $<\theta>$ since the orientation $\theta$ is a circular quantity, which takes on values over a finite range (between $-90^\circ$ and $90^\circ$) that is periodic ($-90^\circ = 90^\circ$). Computing the arithmetic mean can give incorrect average orientations: we should expect the two measurements $-89^\circ$ and $89^\circ$ to average out to $90^\circ$, but the arithmetic mean yields $0^\circ$. In order to accurately determine average orientation $<\theta>$, we first consider the von Mises distribution (a.k.a. circular normal distribution), which is the circular analog of the Gaussian distribution (Jammalamadaka and Sengupta, 2001) (Fig. 22):

$$p(\alpha) = \frac{e^{\kappa \cos(\alpha - <\alpha>)} }{2\pi I_0(\kappa)} ,$$

where $\alpha$ is a circular quantity that varies in the range $[-\pi, \pi]$ and usually corresponds to an angle or phase. The von Mises distribution is parametrized by two parameters: the expectation value $<\alpha>$ and the concentration $\kappa$. These two parameters are analogous to the expectation value $\mu$ and the inverse of the standard deviation $\sigma^{-1}$ of a Gaussian distribution. Note that orientation $\theta$ varies in the range $[-\pi/2, \pi/2]$, whereas angle $\alpha$ varies in the range $[-\pi, \pi)$. Although most circular quantities are measured by an angle $\alpha$ (wind direction, phase of a wave), some physical quantities are rather measured by an orientation $\theta$ (polarization of light, orientation of apolar rods). In order to relate $\theta$ to the von Mises distribution, orientation measurements are multiplied by a factor of 2 to recover angles $\alpha$. The average angle $<\alpha>$ is computed, and then divided by a factor of 2 to recover the average orientation $<\theta>$. The concentration $\kappa$ remains unchanged when converting between orientation $\theta$ and angle $\alpha$.

In Step 5, we use the “CircStat” MATLAB toolbox (Berens, 2009) to compute $<\theta>$ for each pixel (Fig. 23a), which yields the orientation of the nematic director. We also compute the concentration $\kappa$ of the distribution for each pixel (Fig. 23b). In principle, $\kappa$ could act as a measure of the order parameter of the liquid crystal: higher values of $\kappa$ indicate a more sharply peaked distribution. Finally, Fig. 23c shows
the number of orientation measurements per pixel. Most areas are well-sampled ($N \sim 10^2$), but a few restricted sites are under-represented.

**Acknowledgements**

Experimental results and data analysis were obtained in collaboration with Oliver Dammone (U Oxford), Pavlik Lettinga (FZ Jülich) and Dirk Aarts (U Oxford). Bacteriophage fd-virus was kindly provided by Pavlik Lettinga. Simulations and analytical theory were designed and executed by Pieter Mulder, Ioana Garlea, and Bela Mulder (AMOLF). We thank all of these collaborators for fruitful discussions. We furthermore thank Dimitry Lamers, Andries Lof, Gijs Vollenbroek, and Chris Rétif for assistance with microfabrication and electron microscopy. The OrientationJ plugin was written by Daniel Sage (EPFL, Lausanne).